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HypC, the Anthrone Oxidase Involved in Aflatoxin Biosynthesis▽†

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On the basis of gene disruption and enzyme activity, *hypC*, an open reading frame in the region between the *pksA* (*aflC*) and *nor-1* (*aflD*) genes in the aflatoxin biosynthesis gene cluster, encodes a 17-kDa oxidase that converts norsolorinic acid anthrone to norsolorinic acid.

Anthraquinones are natural products of polyketide origin that are found in many organisms, including bacteria, fungi, plants, and insects (1, 6, 11, 16). They are the precursors of the highly toxic and carcinogenic aflatoxins (AFs), compounds found as ubiquitous contaminants of maize, cotton seed, and groundnuts (8). In fungi, anthraquinone-derived polyketides are synthesized by iterative type I polyketide synthases (PKSs) (9), and the expected initial product is an anthrone (Fig. 1). Oxidation is required for formation of the first stable intermediate, for example, the anthraquinone norsolorinic acid in aflatoxin biosynthesis. Oxidation of bacterial anthrones is catalyzed by a 14-kDa monooxygenase that does not require metal ions, prosthetic groups, or cofactors normally associated with oxygen activation (6, 11, 16). Previously, an anthrone oxidase that catalyzed the conversion of emodin anthrone to emodin was isolated from cultures of *Aspergillus terreus* (5, 11), but the gene encoding this protein has not yet been characterized, even though the entire genome of *A. terreus* has been sequenced (12).

In the AF gene clusters from *Aspergillus* section *Flavi* (*Aspergillus parasiticus*, *Aspergillus flavus*, *Aspergillus nomius*, and others), open reading frames (ORFs) are present in the intergenic regions between the *pksA* (*aflC*) and *nor-1* (*aflD*) genes and the *verB* (*aflL*) and *avfA* (*aflI*) genes that are predicted to encode closely related proteins with molecular masses of 17.7 and 17.5 kDa, respectively (7). On the basis of expressed sequence tag (EST) data, these ORFs, *hypC* and *hypB*, are expressed only under conditions conducive to AF production (J. Yu, personal communication). A motif with the canonical binding sequence ($TCGN_5CGA$) for the AF pathway-specific transcription factor, AflR, is located 168 bp upstream from the transcription start point (TSP) of *hypC* and 75 bp upstream from the TSP of *hypB*. In *Aspergillus nidulans*, the sterigmatocystin (ST) cluster contains the ortholog, *stcM*, whose function is not yet known. We now report that *hypC* encodes a monooxygenase similar to the anthrone oxidases found in bacteria and is capable of catalyzing the oxidation of norsolorinic acid anthrone (NAA).

Disruption of *hypC* and *hypB* in *A. parasiticus* BN009E Δ*niaD* was achieved by inserting the nitrate reductase gene (*niaD*) and the pyrithiamine resistance gene (*ptr*), respectively, into the coding region of these genes, using methods previously described (3, 10) (see supplemental material). *A. parasiticus hypC*::*niaD* transformants accumulated excess amounts of NA and a small amount of NAA (Fig. 2A, lane 1). They also accumulated smaller amounts of AFs than transformants with *hypB*::*ptrA* (Fig. 2A, lane 2) or transformants with the *niaD* selection marker only (Fig. 2A, lane 3).

Expression of *hypC* and *hypB* in *Escherichia coli* BL21(DE3)plysS was induced by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to the culture medium as described for expression in *E. coli* of the bacterial anthrone oxidase, AknX (6), except that the *hypC* and *hypB* sequences were totally codon optimized for *E. coli* expression by *in vitro* synthesis (EZ Biolab Inc., Westfield, IN) (see supplemental material). A mutant with Trp43 in HypC replaced by Ser ($HypC_M$) was created by overlap extension PCR using mutant oligonucleotide primers and codon-optimized DNA as the substrate. N-terminally His-tagged HypC, HypB, and $HypC_M$ were isolated from 6 M guanidinium chloride extracts of the insoluble fraction of IPTG-induced *E. coli* lysates by batchwise affinity purification on Ni-nitrilotriacetic acid (Ni-NTA) agarose (15). Both this protein fraction and the soluble fraction obtained as described previously (4) were tested for enzyme activity without further purification.

For enzyme assays, synthetic NAA was prepared from NA by $SnCl_2$ reduction in concentrated hydrochloric acid, a procedure that was used for preparation of emodin anthrone from emodin (13). Liquid chromatography–negative-ion mass spectrometry (LC-MS) confirmed that the product mix contained

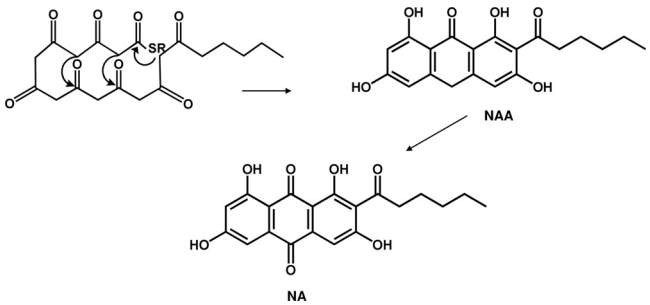

FIG. 1. Schematic diagram showing oxidation of norsolorinic acid anthrone (NAA) formed after aromatic condensation of the polyketide. SR, coenzyme A.

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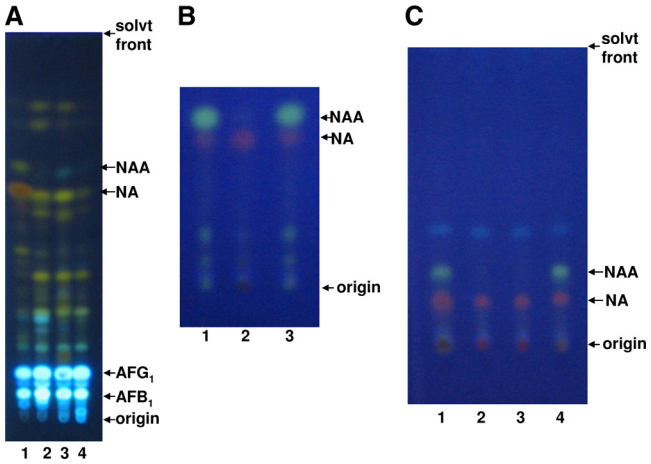

FIG. 2. Thin-layer chromatography (TLC) analyses to demonstrate the function of HypC as an NAA oxidase. (A) Acetone extracts of *A. parasiticus* BN009E *hypC*::*niaD* (lane 1), *A. parasiticus* BN009E *hypB*::*ptrA* (lane 2), *A. parasiticus* BN009E *norA*::*niaD* (lane 3), and untransformed *A. parasiticus* BN009E (Δ*niaD* Δ*ku70*) (lane 4). The plate was developed with toluene-ethyl acetate-acetic acid (8:1:1). The metabolites were identified by comigration with authentic standards. Abbreviations: solvt front, solvent front; AFG_1, aflatoxin G_1; AFB_1, aflatoxin B_1. (B) Products obtained after 3 h of incubation at 37°C of NAA with aliquots of Ni-NTA-purified protein from guanidinium hydrochloride (6 M) extracts of IPTG-induced *E. coli* cultures transformed with pET28a (empty vector) (lane 1), pET28a::*hypC* (lane 2), or pET28a::*hypB* (lane 3). The plate was developed with toluene-acetone (10:1). (C) Incubation of NAA with unpurified soluble protein extracts of IPTG-induced *E. coli* cultures transformed with pET28a (lane 1), pET28a::*hypC* clone 1 (lane 2), pET28a::*hypC* clone 2 (lane 3), or pET28a::$hypC_M$ (mutant *hypC*) (lane 4). The plate was developed with toluene-acetone (10:1).

mainly NAA [*m/e* negative ion = 355 $(M-H)^-$] and a small amount of NA [*m/e* negative ion = 369 $(M-H)^-$]. The UV spectrum of this product mixture had an absorbance maximum (ethanol) at 374 nm similar to that of emodin anthrone. Following the assay conditions reported by Chung et al. (6), partially purified HypC expressed in *E. coli* was incubated with NAA for 1 hour at 37°C in 0.5 M potassium phosphate (pH 5.8), and the metabolites were analyzed by thin-layer chromatography (TLC). Under these conditions, HypC (Fig. 2B, lane 2) caused almost complete oxidation of NAA, while HypB had no effect (Fig. 2B, lane 3). Incubation with the soluble fraction from HypC expressed in *E. coli* gave similar results; extracts from two different *E. coli* clones expressing HypC were able to completely oxidize NAA to NA and other products (Fig. 2C, lanes 2 and 3), but neither the extract from *E. coli* transformed with the empty vector (Fig. 2C, lane 1) nor the extract from cells expressing $HypC_M$ (Fig. 2C, lane 4) were able to oxidize NAA. Similar results were seen using emodin anthrone as a substrate (see supplemental material).

The abilities of the Δ*hypC* mutants to produce AFs and to accumulate NA suggest that nonenzymatic oxidation is a significant alternative pathway allowing loss of the enzyme to be bypassed. Under the conditions of the assay, NAA is slowly oxidized to NA and other products with complete oxidation by 24 h. The latter products were characterized by mass spectrometry as dimeric oxidation products (see supplemental material) related to those found after chemical oxidation of emodin anthrone (2, 14).

A tBLASTN search of the *Aspergillus* comparative genome database (http://www.broadinstitute.org/annotation/genome/aspergillus_group/MultiHome.html) and the multi-fungi BLAST database (http://www.broadinstitute.org/cgi-bin

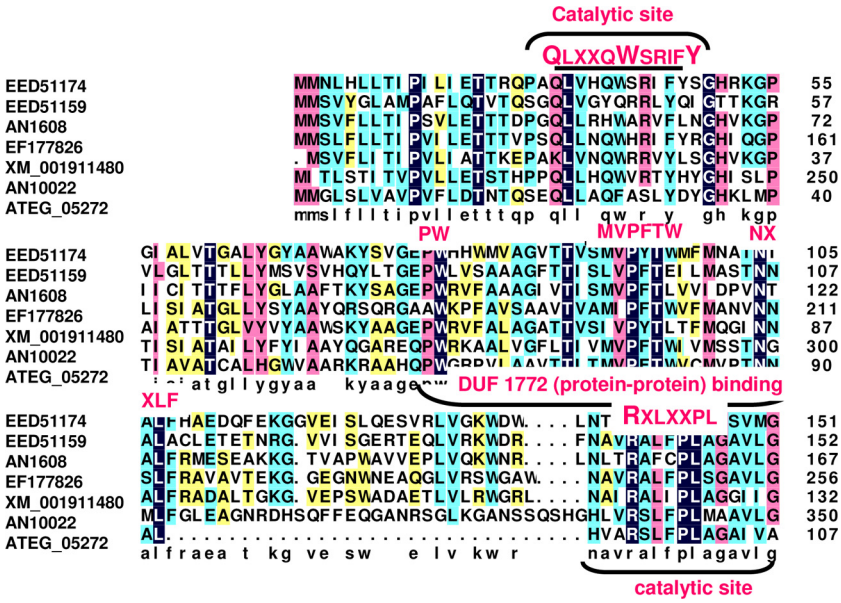

FIG. 3. Alignment of HypC orthologs from different fungi. The sequences of *A. flavus* HypC (EMBL accession no. EED51174), *A. flavus* HypB (EMBL accession no. EED51159), *A. nidulans* ST cluster StcM (*Aspergillus* Genome Database accession no. AN1608), *Mycosphaerella pini* dothistromin cluster hypothetical protein DS12 (GenBank accession no. EF177826), *Podospora anserina* hypothetical protein PODANSg8557 (GenBank accession no. XM_001911480), *A. nidulans* MdpD cluster hypothetical protein (*Aspergillus* Genome Database accession no. AN10022), and *A. terreus* emodin cluster hypothetical protein (Central *Aspergillus* Data Repository accession no. ATEG_05272) are shown. Only partial sequences are shown. Putative catalytic site and other sites are shown above and below the alignment in fuchsia.

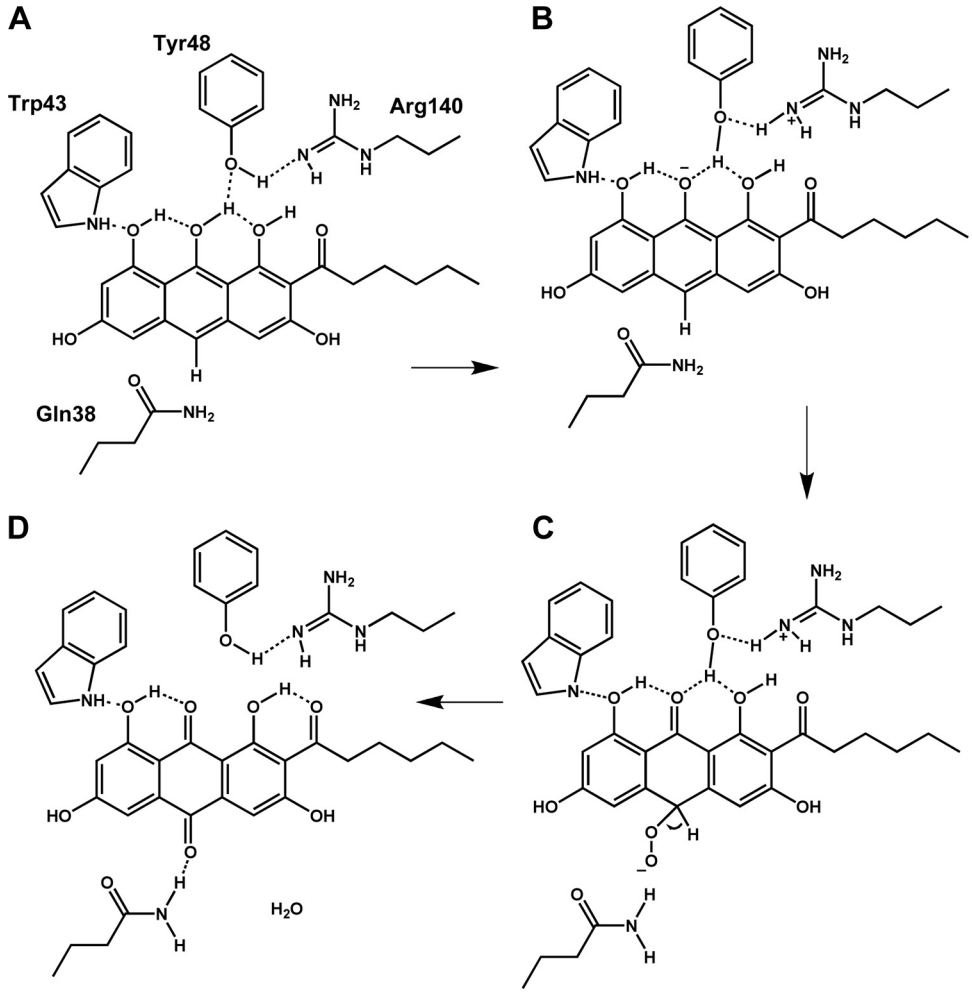

FIG. 4. Putative catalytic mechanism of NAA oxidation. The model is based on the catalytic mechanism suggested by Sciara et al. for oxidation of 6-deoxydihydrokalifungin, an actinorhodin precursor, by ActVA-Orf6 (16). In the model, hydrogen bonding of Trp43 stabilizes the tautomeric form of the anthrone and facilitates proton transfer from NAA to Tyr48 with participation by Arg140 (A) to give a zwitterionic intermediate (B), thereby allowing attack by molecular oxygen at the anthrone secondary carbon (C). (D) The resulting peroxy intermediate is stabilized by hydrogen bonding to Gln38, which loses water to give the anthraquinone.

/annotation/fgi/blast_page.cgi) and PubMed nonredundant protein sequence database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) containing other sequenced fungal genomes revealed that fungi capable of producing products derived from anthrone precursors (sterigmatocystin, monodictyphenone [Mdp], *A. nidulans*; dothistromin, *M. pini*; emodin, *A. terreus*) have genes predicted to encode HypC orthologs in their biosynthesis gene clusters.

Structural studies of ActVA-Orf6 determined that a Trp (W) residue is critical for catalysis of anthrone oxidation (6, 16). Comparison of the predicted amino acid sequences of these HypC orthologs (Fig. 3) located a potential catalytic region that is somewhat similar to the catalytic site for oxidation by some bacterial anthrone oxidases (16). Like the bacterial enzymes, the catalytic regions also contained Gln, Trp, and Asn residues that were able to tether the substrate, while proton transfer is mediated by an Arg in the motif, LRAL. However, this motif is 20 amino acids (aa) closer to the C-terminal end than the similar motif in the bacterial enzyme (16). While there are several candidate Trp residues in HypC, the one that fits best with the Trp residue in the catalytic site, NVAEWRDLASFR, of *Streptomyces galilaeus* aklanonic acid anthrone monooxygenase (AknX [UniProtKB accession no. Q9L552]) and NYAQWESEQAY of *S. coelicolor* ActVA-Orf6 monooxygenase (UniProtKB accession no. Q53908) is Trp43 in HypC in the sequence Q_{38}LVHQW$_{43}$SRIFY$_{48}$SGHR. In HypC, Gln38 would be expected to play a role equivalent to that of Asn in the bacterial oxidases. Furthermore, HypC has an Arg residue at position 140 in the sequence RAL that may serve a function similar to that of Arg86 in ActVA-Orf6 and Arg87 in AknX (also in the sequence RAL). Other Arg residues (Arg52, Arg124, or Arg131) might also be positioned for proton abstraction as shown in the schematic representation of the catalytic site (Fig. 4). In this model, in analogy to the catalysis by ActVA-Orf6, Trp43 serves to facilitate tautomerization of the anthrone which allows proton transfer to Arg140 (Fig. 4A and B). The resulting anion in Fig. 4C reacts with molecular oxygen to form the peroxy intermediate, which, after protonation and loss of water, gives the anthraquinone. The

domain of unknown function (DUF 1772) in HypC and its orthologs identified in tBLASTN searches of the genome databases may mediate protein-protein interaction. Bacterial anthrone oxidases are known to form dimers for catalysis, and DUF 1772 regions could serve as both the interface for catalysis and the domain for dimerization in the fungal enzyme. This region could also be part of the ferredoxin-like fold found in a number of similar oxidases. Therefore, these assignments of catalytic function for HypC are consistent with the proposed catalytic function of the bacterial enzymes. The different spacing of amino acids may be necessary for the enzyme to interface with the hydroxyl residues in the A- and C-rings of NAA.

HypB, while also possessing several Trp residues, is missing the Trp residue in the predicted catalytic motif. The lack of Trp at this position may explain why HypB is unable to catalyze NAA oxidation. Since the sterigmatocystin (ST) biosynthesis cluster in *A. nidulans* possesses only a single homolog of *hypC* (*stcM*), HypB may help mediate an oxidation in AF biosynthesis after the formation of ST. We previously found that Δ*hypB* mutants of a strain of *A. parasiticus* that accumulates only *O*-methylsterigmatocystin (OMST) failed to show any change in metabolite production (J. W. Cary and K. C. Ehrlich, unpublished results). Conversion of OMST to AF requires at least 3 oxidative steps. Only a single enzyme (OrdA and AflQ) has so far been proven to be involved in the conversion, and it is possible that HypB may also be involved in an additional oxidation step.

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